Short Communication

Haemophilus parasuis exhibits IgA protease activity but lacks homologs of the IgA protease genes of Haemophilus influenzae[☆]

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ABSTRACT

Haemophilus parasuis, the bacterium responsible for Glässer's disease, is a pathogen of significant concern in modern high-health swine production systems but there is little information regarding the identity or function of its virulence factors. Several important human mucosal pathogens, including the closely related bacterium Haemophilus influenzae, utilize IgA proteases to aid in defeating the host immune response and facilitate disease but it is unknown whether H. parasuis synthesizes any product with IgA protease activity. To investigate potential virulence mechanisms of H. parasuis, we evaluated five strains for their ability to digest purified IgA. Western blotting demonstrated cleavage of swine IgA, but not human IgA1, following incubation with culture supernatants from three strains, two of which are known to cause invasive disease. No genes with homology to the H. influenzae IgA protease genes iga and igaB could be identified in any H. parasuis strain using either PCR or Southern blotting. These results demonstrate that a novel IgA protease produced by some strains of H. parasuis cleaves the swine IgA heavy chain at a site not found in human IgA1.

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1. Introduction

Haemophilus parasuis is the causative agent of Glässer's disease in swine, characterized by polyserositis, arthritis and lameness, meningitis, and death (Rapp-Gabrielson et al., 2006). It additionally causes pneumonia in the absence of systemic disease. Despite its significant impact on swine health and production, little is known about virulence factors of H. parasuis.

The mucosa of the upper respiratory tract is the primary target of H. parasuis colonization and mucosal lesions that develop during infection have been proposed as a means of access to the bloodstream (Vahle et al., 1997). Immuno-globulin A (IgA) plays a key role in the defense of host mucosa by binding to microbes, trapping them and their products in mucus and blocking adhesin–receptor inter-actions (Woof and Kerr, 2006; Brandtzaeg, 2009). In pigs there are two IgA allotypes, IgA[a] and IgA[b] (Brown and Butler, 1994; Brown et al., 1995). These are differentiated by the length of their hinge regions, with the IgA[b] allotype exhibiting an exceptionally short hinge due to a splice-site mutation.

IgA proteases play an important role in defeating the host immune response for a number of human pathogens, including Haemophilus influenzae and species of Neisseria

and *Streptococcus,* and several IgA protease genes have been identified and characterized (Kilian and Russell, 2005). These genes encode post-proline endopeptidases that attack sites specific to the unusually long thirteen amino acid hinge region of the IgA1 subclass produced by humans and other primates. The IgA2 subclass in primates has a hinge of only eight residues that does not include the peptide bonds attacked by IgA1 proteases. The IgA hinge region of most other animals, including pigs, is most similar to the human IgA2 hinge and all are unaffected by bacterial IgA proteases so far described.

It is presently unknown whether *H. parasuis* exhibits IgA protease activity. Genome sequence data have recently become available for two strains, SH0165 (Yue et al., 2009) and 29755 (GenBank Acc.# NZ_ABKM00000000), but neither annotation identifies potential homologs of IgA1 protease genes. In an attempt to better understand the pathogenesis of *H. parasuis* in swine, we examined several strains for swine IgA protease activity and undertook a search for potential IgA protease genes.

2. Materials and methods

2.1. Bacterial strains and genomic DNA

Bacteria used in this study are listed in Table 1. All were cultivated on Casman Agar Base (BBL) supplemented with 1% (w/v) NAD (Sigma) and 5% GIBCO filtered horse serum (Invitrogen) at 37 °C for 48 h. Genomic DNA used for PCR and Southern blots was purified with a commercially available kit (Promega) and quantified by UV spectrophotometry.

2.2. Purification of swine IgA

Secretory IgA was purified from the milk of sows homozygous for IgA[a] or IgA[b]. The whey fraction was recovered following centrifugation at $150,000 \times g$ and immunoglobulins were precipitated in 50% saturated ammonium sulfate. Following centrifugation at $200 \times g$, pellets were resuspended in 0.05 M Tris, 0.5 M NaCl at pH 8.0. IgA was recovered from the second peak of protein eluted following chromatography on 6B Sepharose. Samples were electrophoresed on 4–15% SDS-PAGE gradient gels (BioRad) and visualized with Bio-Safe Coomassie (BioRad).

2.3. IgA protease assay

IgA protease assays were adapted from published protocols (Fernaays et al., 2006). For each strain evaluated,

two colonies picked from Casman agar plates were suspended in BHI liquid medium supplemented with 10 µg/mL NAD and 10 µg/mL hemin and grown at 37 °C and 185 rpm to an A_{600} of 1.0. Bacterial protein production was arrested by the addition of 30 µg/mL chloramphenicol followed by centrifugation at $1200 \times g$ for 10 min to pellet cells. The supernatants were removed and cells discarded. Sterile medium used as a negative control was incubated and processed in an identical manner.

Separate aliquots of culture supernatants from each strain tested were incubated for 18 h at 37 °C and 185 rpm with 10 µg/mL of either swine IgA[a], swine IgA[b], or human IgA1 (AccuSpec) in a final volume of 100 µL. Assays were performed at least twice for each strain using independently obtained culture supernatants. Negative controls, consisting of either sterile medium or 10 µg/mL IgA in either sterile medium or PBS, were incubated under identical conditions.

Following incubation, samples were electrophoresed on 4–15% SDS-PAGE gradient gels (BioRad) and transferred to nitrocellulose membranes. Membranes were blocked with 5% dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST) at room temperature for 1 h, rinsed briefly in TBST, and incubated with a 1:1000 dilution of heavy chain-specific anti-human or anti-swine IgA-HRP (AbD Serotec). Membranes were washed three times with 50 mM Tris, pH 7.5, containing 150 mM NaCl, 0.05% Tween 20 and 0.25% fish gelatin prior to antibody detection using a chemiluminescent substrate kit (Pierce).

2.4. PCR

H. influenzae sequences for the IgA protease gene, *iga*, found in all IgA-protease positive strains, were retrieved from GenBank (Acc.#s M87489–M87492, CAB56789, and NC_000907), aligned and used to design PCR primers for amplification of the entire open reading frame in 800–1000 bp overlapping fragments (Table 2, primer sets 1–8). Regions with a high percentage of sequence identity were selected for primer binding sites to maximize the likelihood of amplification from *H. parasuis*. Average primer pair T_ms range from ~56 to 61 °C. Approximately one third of *H. influenzae* strains possess a second IgA1 protease gene, *igaB*, and the previously reported primers igaBscreen-5′/igaBscreen-3′ (Fernaays et al., 2006) were used here for PCR amplification of *igaB*.

Unless noted otherwise, PCR with genomic DNA was carried out in a PTC-200 thermocycler (MJ Research) using the following cycling conditions: 95 °C for 5 min, 35 cycles

Table 1
H. influenzae and *H. parasuis* strains used in this study.

Species	Strain	Source	Notes
H. influenzae	Rd KW20	ATCC	ATCC 51907; *iga* positive
H. influenzae	ATCC 33391	ATCC	ATCC type strain; *iga* positive
H. influenzae	11P6H	T.F. Murphy	*iga* and *igaB* positive
H. parasuis	ATCC 19417	ATCC	ATCC type strain; virulence uncertain
H. parasuis	MN-H	J. Torrison	Glässer's disease isolate
H. parasuis	2170B	R. Ross	Glässer's disease isolate
H. parasuis	NADC1	S. Brockmeier	Isolated from a healthy pig
H. parasuis	29755	R. Ross	Glässer's disease isolate

Table 2
Primer sequences for amplification of *iga* and *igaB* genes.

Primer set	Primer	Sequence (5′–3′)	Amplicon length (bp)[1]
1	iga-3	ACT TGT AAC CGT ATA TCA AAT TGT GTC C	
	iga-4	GGG GAG CCA CTG TCG CCT AA	943
2	iga-5	ACC TAT GGT ATT GCA GGC ACA CCT	
	iga-6	AGT ACA ACA GTT GAG CGA CCA C	806
3	iga-7	GTG GGC GAT GGT ACT GTT ATC TTA	
	iga-2	GCC CGT ATA GTC AGA ACG TAC ACA	978
4	iga-1	CAA CAA CGA GCG TAT GAA TGG C	
	iga-8	CTG GGT TAT ACA AAT CGT AAC GTC C	1019
5	iga-9	GTT GTA ACT AAA TCC GCC ACA GGT	
	iga-10	TCC CTG TTT CGG AGA CGC TTG AGA	960
6	iga-11	CCT CAA GTG GCT TCT CAA GCG TCT C	
	iga-12	GCG AAC ATA AGT AAA CAC GCC ACC	807
7	iga-13	TTG CGC GAT CTC ACA AGT ACA AAC AC	
	iga-14	CCT GTA AGA CAC AGG GCT TTG TTG	933
8	iga-11	CCT CAA GTG GCT TCT CAA GCG TCT C	
	iga-14	CCT GTA AGA CAC AGG GCT TTG TTG	1450
9	iga-3	ACT TGT AAC CGT ATA TCA AAT TGT GTC C	
	iga-14	CCT GTA AGA CAC AGG GCT TTG TTG	5234
–	igaBscreen-5′[2]	TGC CCG TTA CCG GAT AAA 1 TG	
	igaBscreen-3′[2]	AAC CAC GGG CAC AAA CAG CC	1650

[1] Based on the sequence of the corresponding *H. influenzae* gene.
[2] Reported by Fernaays et al., 2006.

of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation of 72 °C for 7 min. Reactions contained a final concentration of 1.5 mM MgCl$_2$, 200 μM of each dNTP, 1 U of Taq polymerase and 0.5 μM of each primer in a final volume of 50 μL. A previously described PCR that amplifies a universally conserved segment of the 16S rRNA gene (Register and Yersin, 2005) was carried out with all templates as a positive control.

2.5. Probe synthesis and Southern blotting

A DIG-labeled probe covering the 5.2 kb *iga* gene was synthesized using primer set 9 (Table 2) and *H. influenzae* Rd KW20 DNA with a PCR DIG probe kit (Roche) according to the manufacturer's recommendations. A DIG-labeled 1650 bp probe specific for *igaB* was also synthesized as described above using DNA from *H. influenzae* strain 11P6H and the primer set igaBscreen-5′/igaBscreen-3′ (Table 2).

For Southern blots, 3 μg of DNA was digested with *Pvu*II (Invitrogen), whose recognition site is not found within any known *H. influenzae iga* or *igaB* sequence. Membranes were prepared and used for hybridizations as reported previously (Register et al., 1997). Detection with anti-DIG-alkaline phosphatase and CSPD (Roche) was carried out according to the manufacturer's protocol.

2.6. In silico search for IgA protease gene homologs

Currently available genome sequences for *H. parasuis* include a draft sequence for strain 29755 (GenBank Acc.# NZ_ABKM00000000) and a complete sequence for strain SH0165 (Yue et al., 2009; GenBank Acc.# NC_011852). Homology searches of the NCBI nonredundant nucleotide and SwissProt databases were carried out using protein BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

3.1. IgA protease activity in H. parasuis

Prior to conducting IgA protease assays, the integrity and purity of swine IgA was evaluated by SDS-PAGE. Bands of molecular mass consistent with the heavy chain (~60 kDa), light chain (~22–25 kDa) and secretory component (~72 kDa) were apparent for both IgA[a] and IgA[b] (Fig. 1).

Intact heavy chain was detected in all controls for swine or human IgA (Fig. 2A–C, lanes 9–10). Cleavage of both swine IgA[a] and the short-hinged swine IgA[b] variant occurred following incubation with culture supernatants from three of five *H. parasuis* strains tested (Fig. 2A–B, lanes 1–5). A digestion product of ~45 kDa was generated from both allotypes.

Human IgA1 appeared unaffected by incubation with *H. parasuis* culture supernatants (Fig. 2C, lanes 1–5), demonstrating the specificity of the proteolytic activity for swine IgA. No strain of *H. influenzae* cleaved either swine IgA allotype (Fig. 2A and B, lanes 6–8). However, culture supernatants from all *H. influenzae* strains tested caused degradation of human IgA1 (Fig. 2C, lanes 6–8).

Experiments in which aliquots of culture supernatants were serially diluted in PBS prior to the addition of swine IgA provide further support for an IgA protease secreted by some isolates of *H. parasuis*. Results obtained with strain NADC1, which exhibited IgA protease activity in the standard assay, are shown in Fig. 3. The degree of
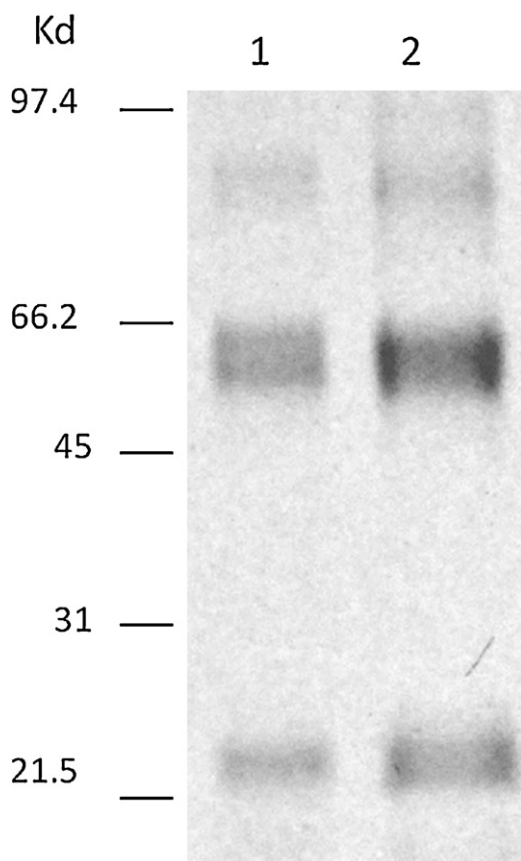
Fig. 1. SDS-PAGE of swine secretory IgA. Purified swine IgA[a] (lane 1) or swine IgA[b] (lane 2) was electrophoresed in a 4–15% acrylamide gel followed by staining with Bio-Safe Coomassie. The positions of molecular mass markers are indicated.

Fig. 2. IgA protease activity in culture supernatants. Analysis of swine IgA[a] (A), swine IgA[b] (B) or human IgA1 (C) following incubation with culture supernatants of the *H. influenzae* or *H. parasuis* strains indicated as detected on Western blots. Lanes: (1) *H. parasuis* ATCC 19417; (2) *H. parasuis* 2170B; (3) *H. parasuis* NADC1; (4) *H. parasuis* 29755; (5) *H. parasuis* MN-H; (6) *H. influenzae* Rd KW20; (7) *H. influenzae* ATCC 33391; (8) *H. influenzae* 11P6H; (9) IgA in PBS; (10) IgA in BHI; (11) BHI only. The position of the swine IgA digestion product is indicated with an arrow. The positions of molecular mass markers are indicated to the left of each panel.

digestion of IgA[a] (panel A) and IgA[b] (panel B) diminished progressively with increasing dilution of the culture supernatant.

3.2. Search for IgA protease gene homologs in H. parasuis

The results detailed demonstrate that some strains of *H. parasuis* produce an IgA protease with a unique substrate specificity. The various IgA proteases so far characterized for *Haemophilus* and *Neisseria* similarly possess unique specificities that are reflected by variable regions in the corresponding genes (Plaut and Wright, 1995). Interspersed among these variable regions are stretches of highly conserved nucleotides that provide a potential basis for recognition of additional IgA protease genes. In an effort to identify IgA protease homologs in *H. parasuis* we carried out PCR and Southern blotting based on the *H. influenzae* IgA protease genes *iga* and *igaB*.

No amplicons were detected in PCRs with any of the five *H. parasuis* strains included in this study using either *iga*- or *igaB*-specific primers, even with annealing temperatures up to 8 °C lower than the average primer pair T_m. Amplicons of the predicted sizes were obtained with all primer sets using the appropriate *H. influenzae* positive
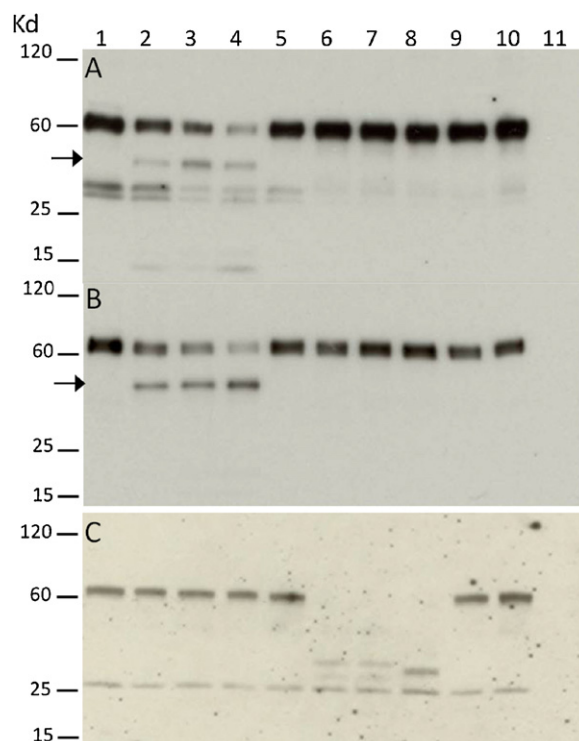
control strains (Table 1; data not shown). All *H. influenzae* and *H. parasuis* strains tested were PCR positive with the universal 16S positive control primer set (data not shown).

Similarly, no IgA protease homologs could be identified in any *H. parasuis* strain using Southern blots hybridized with either an *iga*- or *igaB*-specific probe, even under low-stringency conditions. Fragments were detectable with both probes in all lanes containing DNA from appropriate *H. influenzae* control strains (Table 1) under both high- and low- stringency conditions (data not shown).

No IgA protease genes are identified in the annotated genome of *H. parasuis* 29755 yet this strain possesses swine IgA protease activity. Therefore, we further scrutinized the 29755 genome sequence in an effort to identify potential IgA protease gene homologs. The output of a BLAST search for amino acid similarity between all predicted proteins from strain 29755 and the NCBI nonredundant nucleotide and SwissProt databases consists of 65 ORFs of 29755 whose best match is an entry containing the term "IgA" or "protease." Further examination of this dataset identified ORF 02137 (GenBank Acc.# ABKM01000132.1) as the most likely candidate for an IgA protease-like gene. The ORF in 29755 lies at the terminus of a contig and, as a result, is truncated.
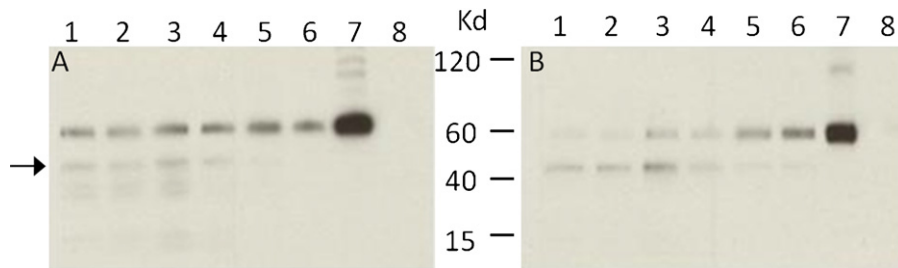
Fig. 3. Swine IgA protease activity of serially diluted culture supernatant. Undiluted supernatant (lane 1) or successive doubling dilutions of supernatant (lanes 2–6) from cultures of *H. parasuis* strain NADC1 were incubated with swine IgA[a] (A) or IgA[b] (B) and samples were subjected to SDS-PAGE and Western blotting. Controls included swine IgA incubated in PBS (lane 7) or BHI medium incubated without added IgA (lane 8). The position of the swine IgA digestion product is indicated with an arrow. The positions of molecular mass markers are indicated between the panels.

However, the homolog in *H. parasuis* SH0165 (GenBank Acc.# CP001321.1) is a complete ORF annotated as a putative extracellular serine protease with an autotransporter region and weak domain homology to Mac-1. An additional but nonidentical copy of the gene is present in SH0165, approximately 3 kb distant and in the opposite orientation. The copy is not found in the current 29755 draft genome sequence but is predicted to lie within a sequence gap.

4. Discussion

This is the first report documenting IgA protease activity in *H. parasuis* and demonstrating an IgA protease with a substrate other than human IgA1. The specificity of the protease for swine IgA suggests a possible role in the pathogenesis of Glässer's disease, in which the bacterium somehow overcomes mucosal host defense mechanisms and spreads systemically. IgA proteases have been implicated in the virulence of various human mucosal pathogens with more invasive strains displaying increased IgA proteolytic activity (Vitovski et al., 1999,2002; Fernaays et al., 2006). However, of the three Glässer's disease isolates evaluated here only two (29755 and 2170B) were able to cleave swine IgA. Little is known about other potential virulence factors of *H. parasuis* but MLST analyses suggest a considerable degree of genetic heterogeneity exists within the species (Olvera et al., 2006) and that strains 29755 and 2170B, contained within the same major clade, are much more closely related to one another than to the remaining Glässer's disease isolate evaluated here, MN-H (Mullins et al., 2009). It may be that genetically divergent strains utilize distinct virulence mechanisms and that alternative bacterial products facilitate the spread of MN-H and other closely related, invasive strains. Evaluation of additional invasive strains will reveal whether IgA protease activity is restricted to those of particular phylogenetic lineages.

Our results also provide the first definitive demonstration of proteolytic activity for swine IgA by any bacterial pathogen. Kilian et al. (1979) reported cleavage of swine secretory IgA by two strains of *Actinobacillus pleuropneumoniae* (classified at that time as *Haemophilus pleuropneumoniae*). However, the method used for preparation of IgA was not detailed and SDS-PAGE of an untreated control revealed a single band of undetermined molecular mass rather than the expected pattern of bands representing heavy chain, light chain and secretory component. Furthermore, the cleavage products were visualized as a diffuse, ladder-like pattern of fragments more suggestive of nonspecific degradation rather than a site-specific proteolytic activity. Using swine IgA purified as described here, this result could not be repeated (M. Kilian and J.E. Butler, unpublished data). Mulks et al. (1984) subsequently reported no swine IgA proteolytic activity in several strains of *A. pleuropneumoniae*, including one studied by Kilian et al. (1979). Others observed weak proteolytic activity against swine IgA using concentrated *A. pleuropneumoniae* culture supernatants (Negrete-Abascal et al., 1994) but this appears to be due to the general activity of metalloproteases that additionally degrade other host proteins rather than the action of a true IgA protease (Negrete-Abascal et al., 1998).

IgA proteases characterized to date cleave specific proline–serine or proline–threonine bonds within the hinge of human IgA1 not present in either allotype of swine IgA. A proline–serine in the swine IgA[a] hinge absent from human IgA1 might be postulated as a cleavage site in that allotype. These residues are missing from swine IgA[b] but an additional proline–serine found in both swine IgA allotypes and not in human IgA1 is present just C-terminal to the hinge. In addition, as compared to human IgA1, there are many amino acid substitutions immediately adjacent to the swine IgA hinge that could provide a host species-specific site of attack for the IgA protease of *H. parasuis*. The size of the digestion fragment generated from both swine IgA[a] and IgA[b], 45 kDa, is consistent with a cleavage site within or adjacent to the hinge or in the C-terminal Cα3 domain of the heavy chain. N-terminal sequencing of the swine IgA digestion products will establish the exact site of cleavage.

Although IgA1 proteases are functionally identical, their activity is mediated through at least three different catalytic mechanisms. Comparison of gene sequences and substrate specificities suggests that the distinct IgA proteases so far characterized arose independently through convergent, function-driven evolution, rather than through lateral gene transfer (Kilian and Russell, 2005). The absence of a recognizable human IgA1 protease homolog in the *H. parasuis* genome similarly implies that the swine IgA protease is unique and evolved independently of other IgA proteases. This is of broad evolutionary relevance since

IgA1, with its exceptional extended hinge, is a biological rarity found only in humans and a few other primates. Substantially greater similarity in the hinge and adjacent regions is shared among the IgAs of most other mammals, including pigs (Mestecky et al., 2005). Thus, the swine IgA protease of *H. parasuis* may belong to a family of proteases that target sites in the hinge or Cα2 domain that are shared among several, or even most, nonprimate mammals.

A role for the *H. parasuis* IgA protease in virulence has yet to be examined but convergent evolution of such a highly specialized catalytic activity strongly argues for its biological significance. IgA1 proteases of other bacteria contribute to virulence not only through cleavage of IgA but also through unrelated activities including destabilization of lysosomes, modulation of T-cell responses and induction of proinflammatory cytokines (Kilian and Russell, 2005). Future efforts focusing on purification and characterization of the swine IgA protease and identification of the gene(s) involved will facilitate our understanding of its role in localized and disseminated disease resulting from *H. parasuis* infection of pigs.

Acknowledgements

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